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Immunophenotyping of Acute Leukemias – From Biology to Clinical Application

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Abstract

Immunophenotyping is an essential part of the modern diagnostic workup of acute leukemias and thus for an appropriate treatment of these complex and heterogeneous diseases. It provides a lot of useful information in this setting that transfers directly from laboratory to clinical management of patients. Lineage definition is the first goal leading to proper initial therapy. Some phenotypic patterns define specific subsets correlating with poor (mixed phenotype, dendritic cell neoplasm) or favorable (cortical T-lymphoblastic leukemia) outcome, thus guiding the application of treatment modalities. An advanced analysis of phenotypic data can address specific issues, such as the still debated role of multilineage dysplasia. The quality of response to chemotherapy is monitored by the detection of minimal residual disease and peripheral blast clearance during chemotherapy delivering. That allows a sharp discrimination of prognosis and again can drive the intensity of therapies proportionally to the disease chemosensitivity.

Keywords: Acute myeloid leukemia, acute lymphoblastic leukemia, diagnosis, prognosis, chemosensitivity, minimal residual disease

1. Introduction

1.1. Definition and clinical picture

Acute leukemias (AL) are hematological neoplasms featured by altered proliferation and/or differentiation of hematopoietic progenitors, leading to accumulation of immature cells in bone marrow (BM) and peripheral blood (PB). The clinical consequences are thus due to BM failure and infiltration of extra-hematological sites by leukemic cells, possibly causing organ function impairment. BM failure and related reduction in peripheral mature elements are responsible for the majority of clinical signs and symptoms at disease onset, that is, fatigue,

skin pallor, tachycardia (due to anemia), infections (due to neutropenia and/or T-cell deficiency), and hemorrhage (due to thrombocytopenia and/or coagulopathy). The clinical spectrum can be very wide, ranging from complete lack of manifestations to life-threatening ones. As such, a correct diagnosis is the first step for the right management of patients affected by AL.

1.2. Principles of treatment

Overall, treatment of AL goes through two main phases: induction and consolidation. The primary target of induction phase is the achievement of a complete remission (CR), which is defined by the decrease of immature cells below 5% of global BM cells at morphologic evaluation [1]. The benchmark of induction is chemotherapy, with different drugs, dosages, and schedules, depending on lineage definition and further on subclassification within different lineages. Some specific subsets [i.e., acute promyelocytic leukemia, Philadelphia-positive acute lymphoblastic leukemia (ALL)] benefit from the application of therapies targeted toward their unique underlying molecular pathways of leukemogenesis. Once more, the delivery of an appropriate treatment from the outset strictly depends upon AL's precise diagnostic definition. CR attainment is a prerequisite for long-term survival; once obtained, the consolidation phase deals with lowering the risk of relapse. Consolidation strategies include allogeneic hematopoietic stem cell transplantation (HSCT), providing the highest impact on relapse risk, chemotherapy, or autologous transplant. The use of allogeneic HSCT is generally reserved to patients with high relapse risk or with refractory disease, given the relevant treatment-related toxicity and mortality. The appraisal of prognosis at disease onset and during treatment phases is a dynamic and challenging process, with many potential predictors in turn characterized by different effectiveness in different contexts. Again, this process is the driver of important clinical decisions involving the application of highly toxic therapies.

1.3. Role of immunophenotype

Immunophenotyping is an essential part of the modern diagnostic workup and prognostic stratification of AL and thus for an appropriate treatment of these complex and heterogeneous diseases. It provides a lot of useful information in this setting that transfers directly from laboratory to clinical management of patients.

2. Diagnosis

According to the World Health Organization (WHO), ALs are defined as hematological neoplasms featured by the presence of 20% or more immature cells (blasts) with respect to PB or BM total cells [2, 3]. This benchmark threshold refers to morphology, which allows to recognize blasts by specific immature properties of nucleus and cytoplasm. Similarly, immunophenotyping permits to identify immature cells based on some immunological characteristics and, specifically, a dim expression of CD45 with low/intermediate side scatter signal [4]. Once identified, blasts are analyzed as regards the expression of a group of core

antigens. The phenotypic profile of blasts is thus related to normal hematopoietic counterpart, to define main lineage of differentiation and subclassification.

2.1. AL lineage definition

The first and main purpose of AL immunophenotyping is lineage definition of blasts, as myeloid or lymphoid, and in the latter case as B or T lymphoid. Lineage definition is based on similarities in antigenic expression patterns to normal myeloid, B-lymphoid progenitors, or T-lymphoid progenitors. With this respect, some “key” antigens have been defined for each lineage based on early and exclusive expression within them. However, it was immediately evident that in some AL forms, there was no clear-cut distinction and cross-lineage expression was quite frequent. As such, the first systematic attempt to regulate these findings was based on the concept that different antigens had a different weight for lineage attribution and was done by European Group for Immunological Classification of Leukemia (EGIL) [5] (Table 1). Although rigid by definition, as any such classification, it had the fundamental merit of creating a common reference for AL diagnosis. Recently many efforts have been tried to ameliorate and fine-tune the basic concept of EGIL classification. On the one side, the core group of lineage antigens has remained substantially the same: the interpretation of their pattern for lineage attribution has been updated by the WHO 2008 Classification [6]. On the other side, main efforts have been pursued to overcome the mere application of thresholds of antigen positivity, based on scarce biologic plausibility, and to attain a higher level of standardization. On this regard, the EuroFlow group has obtained amazing results toward sample handling, optimization on combinations of monoclonal antibodies, and even data analysis, possibly heading to go beyond dependence on operator [7].

Score	Myeloid	B lymphoid	T lymphoid
2	cyMPO	CD22, CD79a, cyIgM	cyCD3, TCR
1	CD13, CD33, CD117, CD65	CD19, CD10, CD20	CD2, CD5, CD8, CD10
0.5	CD14, CD15, CD64	CD24, TdT	TdT, CD7, CD1a

Abbreviations: MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase; cy, cytoplasmic; TCR, T-cell receptor.
 More than 2 points are required to assign a lineage.

Table 1. Classification of European Group for Immunological Classification of Acute Leukemias

2.2. Diagnosis in challenging contexts

For an immediate diagnostic assessment, in the majority of cases, morphology is already able to assign myeloid lineage mainly by revealing granules, Auer bodies, or monocytic features. However, in several circumstances, immunophenotyping is absolutely essential for diagnostic workup:

- Morphologically undifferentiated blasts: when clear myeloid features are absent in PB- or BM-stained slides, immunophenotyping is the cornerstone of AL diagnosis. That pro-

vides the basis for definition of myeloid or lymphoid leukemia, a definition leading to completely different treatment strategies. In turn, within lymphoid lineages, phenotypic pattern is the basis for subclassification, according to the expression of selected key antigens in parallel with the maturation process in normal lymphopoiesis (Figure 1).

- Atypical presentation: ALs sometimes have atypical behavior, making hard to get a diagnosis and thus adequate therapy. These relatively rare circumstances are as follows: cases with low peripheral blast count and *punctio sicca* at BM aspiration; aplastic onset of T-lymphoblastic leukemia; BM necrosis reported to be associated with ALL or NPM1-mutated acute myeloid leukemia (AML) [8].
- Neoplasm of precursors of plasmacytoid dendritic cells: it is a novel subset in the WHO classification of myeloid neoplasms, with heterogeneous clinical presentation and variable extra-hematological infiltration. The normal counterpart of this tumor resides in the plasmacytoid dendritic cell lineage and as such its diagnosis depends on revealing a dendritic cell-related phenotype [9, 10]. The typical phenotypic profile consists of positivity for CD4, CD56, and CD123, together with intense expression of HLA-DR and negativity for main lineage antigens. In this setting, the phenotype provides an important piece of clinical information since this disease has a dismal outcome with chemotherapy consolidation and allogeneic transplantation seems to provide better chances of cure [11].

Figure 1. Subclassification of acute lymphoblastic leukemia in parallel to normal lymphopoiesis. Acute lymphoblastic leukemia of B lineage (panel A) and of lineage T (panel B) is classified according to phenotypic profile of blasts and its similarity to the stages of physiological lymphopoiesis.

2.3. Immunotherapy of AL

The application of immunotherapy is an emerging treatment approach in the field of AL. The basic concept is the killing of leukemic cells by direct attack to molecules on the cell surface. B-lineage antigens, CD19 and CD22, are effectively targeted by rituximab and inotuzumab, respectively, in B-ALL. Gemtuzumab ozogamicin is directed to pan-myeloid antigen CD33 and it is used in AML. More recently, immunological therapies based on the activation of T lymphocytes directly against blasts have been developed in B-ALL. This approach relies on bispecific T-cell engager antibodies, such as blinatumomab [12], or on autologous T lymphocytes engineered with chimeric activating receptors (CAR) [13] and represents one of the most promising strategies in the therapy of this disease. It is obvious that the application of immunotherapy relies on revealing the expression of the target antigens on the membrane of pathologic cells and thus on proper immunophenotyping.

3. Correlation phenotype–genotype

Karyotype and molecular genetics still represent the cornerstone of prognostic stratification of patients affected by AL. This information commonly drives the application of treatment. In AL bearing recurrent cytogenetic abnormalities, leukemic cells often display shared antigenic patterns usually able to predict the underlying genotype. Generally, this association is strong when few and relevant genetic events are responsible for leukemogenesis [i.e., CBF-AML, *t*(15;17), Philadelphia chromosome], whereas it is weaker with higher genetic heterogeneity (i.e., normal/intermediate karyotype with NPM1, FLT3, CEBPA, DNMT3A, IDH1-2 gene mutations). The definition of such “predictive” profiles is based on entire phenotypic patterns of blasts rather than on single antigen expression [14].

t(8;21): AML with *t*(8;21) is typically featured by maturation asynchrony, that is, high expression of CD34 together with mature antigens as CD15 and myeloperoxidase. Most cases display a cross-lineage co-expression of “dim” CD19. TdT and CD56 are common aberrancies on blasts. Maturing neutrophil compartment often shows phenotypic abnormalities (lack of CD10, expression of CD56) as well, due to its belonging to leukemic clone.

inv(16): often associated with eosinophilia, blasts of this AML subset show co-expression of immature antigens concomitantly to markers of granulocyte (CD15, CD65) or monocyte differentiation (CD14, CD4). In approximately half of cases, a cross-lineage expression of CD2 is observed.

NPM1: AML with mutations in the NPM1 gene usually shows monocytic differentiation. When detectable, myeloid blasts are often featured by negativity or dim expression for CD34 [15]. Typically, monocytic cells display immature phenotypic profile.

t(15;17): acute promyelocytic leukemia has two main characteristics: life-threatening coagulopathy and sensitivity to a differentiating treatment consisting of all-trans retinoic acid. Both of them prompt the urgency of a correct suspect and consequent diagnosis. In this light, the interpretation of the phenotypic profile of blasts is crucial. Leukemic promyelocytes typical-

ly have the phenotype of their normal counterpart (i.e., high side scatter signal with intense expression of CD33 and CD64, heterogeneous expression of CD13, negativity for HLA-DR) but with dim/negative CD15 [16]. Cross-lineage expression of CD56 can occur, with debatable prognostic meaning. A population of basophils with extremely high side scatter signal (SSC) can also be revealed.

t(9;22): this chromosomal aberration causes a chimeric fusion gene called BCR/ABL, a crucial event in leukemogenesis. Within ALs, the majority of cases bearing this translocation are B-precursor ALL and, frequently, are featured by CD10 expression. Moreover, most cases display several aberrant features. Among them, the expression of myeloid antigens (CD13 and/or CD33) is frequent as well as the expression of CD25 (interleukin-2 receptor alpha chain). Rather than a single aberration, the whole phenotype of blasts can reliably predict an underlying *t(9;22)*, as reported by Tabernero et al. [17].

In the field of genotype prediction, a novel application of flow cytometry (FC) based upon an immunobead assay has been developed. This kind of test is able to detect the presence of chimeric proteins coded by relative fusion genes such as PML/RAR α and BCR/ABL and, obviously, has the advantage to provide earlier results compared to PCR [18, 19].

Due to the capability to provide early results, immunophenotype is extremely useful to guide the analysis of karyotype and molecular genetics, aiding the application of the FISH method, for example. In this context, immunophenotyping can have a crucial role when karyotypic analysis results in lack of growth. The phenotypic profile can reliably channel genetic analyses toward most probable mutations, thus tuning an otherwise undefined prognostic assessment.

4. Prognostic value at baseline

As described above, immunophenotyping has mainly a diagnostic role in AL field. At any rate, there are some specific, less common subsets where FC assumes a significant prognostic weight, upon which clinicians can apply a proportional treatment strategy. These subsets are as follows:

4.1. Mixed Phenotype (MP) AL

The concomitance of expression of antigens belonging to different lineages has demonstrated to correlate with dismal prognosis. Consistently, the WHO classification considers these cases as a separate entity. The diagnostic criteria in the WHO 2001 classification adopted those previously defined by EGIL (see Table 1): a score >2 points for two or more lineages defined biphenotypic AL [5]. The WHO 2008 classification has established new criteria for the diagnosis of this subset, also renamed as MP-AL. Highly lineage-specific antigens are required for T- (cyCD3) and myeloid (myeloperoxidase and/or evident monocytic differentiation) lineage attribution. For B-lineage, one or three B-lineage markers have to be expressed depending upon intense or weak expression for CD19 [6]. MP-AL is usually associated with unfavorable karyotype, MLL/11q23 rearrangements, or BCR/ABL gene fusion. Patients

affected by MP-AL are characterized by unfavorable prognosis and should be considered for allogeneic HSCT once in CR [20].

4.2. Cortical T (EGIL T-III) acute lymphoblastic leukemia

This subset is defined by the expression of CD1a (see Figure 1). In a large multicenter prospective trial by UKALL and ECOG, a lower relapse risk and a longer overall survival were observed for this category of patients [21]. This immunological feature is often used for prognostic stratification of T-ALL.

4.3. Early T (ETP) ALL

A specific immature T phenotype, featured by absence of CD1a and CD8, weak CD5, and expression of one or more myeloid or stem cell-related antigens, has been associated with low response rate to chemotherapy and dismal prognosis [22]. However, the prognostic analyses within more modern clinical trials have shown survival similar to non-ETP ALL and the prognostic meaning of this subgroup is still under debate [23].

5. Advanced phenotypic analysis: assessment of multilineage dysplasia

Several papers have addressed the role of multilineage dysplasia (MLD) in AML leading to conflicting results, possibly because of technical and biological reasons [24, 25]. Technical reasons deal with morphological assessment of residual hematopoiesis at AML diagnosis, that is the standard criteria for defining MLD. Morphology is operator dependent; its specific application is even more complicated in this setting because residual nonblast cells are very few at diagnosis. Biologically, the MLD-related unfavorable prognosis would rely on clonal involvement at stem cell level or on pre-existing clonal hematopoiesis, conferring bad prognosis to AML. However, MLD might merely result from pathologic differentiation/maturation of the leukemic clone. We thus estimated MLD by FC, which is emerging as a useful method to study dysplasia, mainly by investigating the expression of key antigens throughout myeloid maturation. Our rationale was that an FC-based evaluation of dysplasia could get further insight into MLD actual significance. The application of FC to study BM maturing cell compartments in AML can provide many advantages compared to morphology: i) the amount of studied cells is much larger; ii) phenotypic parameters can be quantified and referred to control groups and, as such, reliably standardized; iii) phenotypic scores can be calculated, thus estimating accurately the extent of dysplasia; iv) there exists a capability to reveal dysplasia even in the absence of atypical morphology in MDS, as shown in a recent report [26]. Technically, the appraisal of MLD was based on a group of control BMs used to set normal phenotypic profile of maturing (i.e., neutrophil and erythroid) compartments; phenotypic abnormalities in AML were thus highlighted by reference to controls and the degree of dysplasia was appraised by a score proportional to deviation from normal phenotypic profile.

We focused our analysis on NPM1-mutated AML, for which a major controversy in the WHO classification exists. In fact, it is still under debate how to classify cases presenting with MLD

and a concomitant NPM1 mutation. This issue has relevant implications since NPM1-mutated status correlates with a relatively good prognosis (especially when FLT3-wt) [27].

Our study provided evidence that MLD, as assessed by immunophenotype, has no impact on clinical characteristics and outcome in NPM1+ AML. By investigating NPM1 status on separated cell compartments, we have established a correlation between MLD and belonging to AML clone [28] (Figure 2). Together with previous reports [29, 30], our findings further support MLD to be part of the spectrum of NPM1+ AML, without any relevant influence on major disease features and outcome. As such, these data strongly suggest the classification and the prognostic stratification of this category of patients should not be based upon MLD.

Figure 2. NPM1 mutational analysis on sorted cell fractions. Cell compartments are shown at the top; as concerns neutrophil compartment (left), the results of clustering analysis are depicted, together with phenotypic parameters and compartment's phenotypic score (IPS), appraising the extent of dysplasia. In the corresponding plots, the cell population is highlighted by color: blue for neutrophil cells, red for blasts, orange for T lymphocytes. The relative data from NPM1 mutational analysis are reported below. (A) Patient #1: neutrophil compartment showed several phenotypic abnormalities (as represented by clustering analysis and IPS = 6.0) and harbored NPM1 mutation; sorted blasts and T lymphocytes were NPM1-mutated and wild-type, respectively. (B) Patient #2: neutrophil compartment showed a preserved phenotypic profile (IPS = 0) and a NPM1 wild-type status. Sorted blasts and T lymphocytes were NPM1-mutated and wild-type, respectively. Clustering analysis was performed by R software. Dot plots were created by Infinicyt software. NPM1 mutational analysis: in order to discriminate NPM1 PCR products, we used 5'-end HEX dye-labeled reverse primer (M-Medical). The amplified products were separated with a capillary electrophoresis-based system using ABI PRISM 310 genetic analyzer (Applied Biosystems). The labeled fragment size corresponding to NPM1 wild-type gene was 347 bp. All NPM1-mutated samples were heterozygous, showing a double peak at positions 347 (wt) and 351 (mut).

6. Chemosensitivity assessment: minimal residual disease and peripheral blast clearance

Identification of AL patients who would have high likelihood to respond to standard induction therapy and those with low probability to do well and those who are candidates for more aggressive treatment is of major clinical importance. Most clinical and biological prognostic factors are based on characteristics of the patient and the disease at diagnosis and are surrogate for disease's chemosensitivity [31, 32]. In AML, the European Leukemia Net (ELN) stratification system is one of the most adopted systems and is based on cytogenetic/molecular abnormalities [31]. In fact, it allows to define patients' subgroups featured by high likelihood to achieve CR and long survival (ELN-favorable) and at the opposite a category with scarce response to chemotherapy and dismal prognosis (ELN-adverse). However, in the absence of genetic determinants, the ELN system merges patients with heterogeneous diseases (intermediate-1 and 2), where its clinical utility has major concerns. The same issue regards the field of ALL. The actual BM response to chemotherapy allows to refine the pretreatment risk stratification as it expresses the actual chemosensitivity resulting from killing leukemic cells.

6.1. Minimal Residual Disease (MRD)

Within responding patients, the detection of MRD beyond morphologic definition of CR is emerging as an accurate tool to refine risk category assignment, as initially established upon cytogenetic/genetic findings. FC allows to study MRD in the vast majority of patients (about 85% in AML, 90–95% in ALL), which is an advantage compared to molecular techniques.

The core concept of MRD by FC is the detection of one or more leukemia-associated aberrant immunophenotypes (LAIP), as phenotypic profiles that are absent or very rare on normal cells [33–35]. The sensitivity of MRD by FC ranges between 10^{-3} and 10^{-4} . MRD is usually estimated as the percentage of LAIP+ cells on global BM cells at certain time points. An alternative approach exploits logarithmic decrease of LAIP+ cells from diagnosis to BM assessment [36]. Conventionally, MRD is evaluated upon full recovery of PB counts after one and/or two chemotherapy cycles [33, 37]. An earlier BM morphologic and FC evaluation (7 days after completing induction) has been shown to correlate with CR achievement and survival [36]. Also at later time points in the treatment plan, for instance, before allogeneic HSCT, MRD confirms its prognostic weight [38].

In ALL, and especially in childhood, MRD by immunophenotype has been embedded within main clinical trials and is used to drive treatment strategy [39]. In AML, in spite of a clear prognostic meaning, MRD by FC has not found a defined place in clinical practice yet. The only published trial basing a clinical decision on MRD regarded the field of childhood AML [40]. Main reasons of this discrepancy between ALL and AML reside probably in a higher phenotypic heterogeneity of both normal and leukemic myeloid hematopoiesis compared to lymphopoiesis and ALL. Strictly linked to this subject, a substantial lack of standardization in method and data interpretation is still a major concern of FC in MRD setting, compared to molecular techniques.

6.2. Peripheral Blast Clearance (PBC)

Early response to treatment, and specifically to steroids, has been established as an important prognostic factor in ALL [41]. In modern therapy, this parameter is appraised by clinicians, but it is not generally considered *per se* as a driver of treatment modalities.

In AML, risk-oriented treatment exclusively concerns post-induction phase. As said before, the disease’s characterization at diagnosis and the quality of response to therapy (estimated by MRD) are integrated to define a prognostic assessment that guides the consolidation phase. In fact, induction is delivered irrespectively of patient or AML features.

Figure 3. Relationship between peripheral blast clearance (PBC) during induction therapy and bone marrow response in AML. (A) PBC promptly resolves responders (CR) from nonresponder (NCR) patients. Log reduction is the ratio between baseline and daily absolute LAIP+ blast count converted to a logarithmic scale. The ranges of log reduction show minimal overlap between the two groups. Horizontal bars are medians, boxes are 25th percentiles, and whiskers are 75th percentiles. Dots are outliers. (B) Bone marrow blast clearance correlates with PBC. In this graph, the log decrease in bone marrow LAIP+ blasts (assessed by flow cytometry on day 14) is in linear relationship to log reduction of LAIP-positive blasts from peripheral blood at day 5 of induction treatment. In fact, a linear statistically significant correlation is found as from day 2.

To assess AML chemosensitivity in a relevant clinical time, we have evaluated the kinetics of leukemic cell reduction from PB during induction. To do this, we have quantified LAIP+ cell population on PB immediately before and daily during induction course. The ratio between absolute LAIP+ baseline and daily values converted to a logarithmic scale was defined as peripheral blast clearance (PBC).

We carried out a daily quantitative assessment of peripheral blasts during conventional “3+7” course in a cohort of 61 patients. We documented that PBC strictly correlated with the decrease of the overall leukemic burden in the patient. Specifically, we observed that PBC discriminated between responsive and refractory patients since day 2 of therapy [42, 43] (Figure 3). Being a very early and powerful predictor of CR achievement and outcome, PBC could allow to modulate the intensity of treatment since induction phase, providing an *in vivo* chemosensitivity assay in AML.

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